UNIVERSITI PUTRA MALAYSIA

THE EVALUATION, PRODUCTION, AND DERIVATISATION OF THE GARCINIA ACID IN GARCINIA ATROVIRIDIS

HABIBAH ZAINAL ABIDIN.

IB 2005 10

THE EVALUATION, PRODUCTION, AND DERIVATISATION OF THE GARCINIA ACID IN *GARCINIA ATROVIRIDIS*

By

HABIBAH ZAINAL ABIDIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of Requirement for the Degree of Master of Science

June 2005

DEDICATION

SPECIALLY DEDICATED TO:

***My husband ABD* RAHMAN BIN BAIN**
'THANKS FOR YOUR UNDERSTANDING AND SUPPORT'

My son **AHMAD IRWAN BIN ABD RAHMAN**
'THANKS FOR YOUR SACRIFICE'

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in the fulfilment of the requirement for the Degree of Master of Science

THE EVALUATION, PRODUCTION, AND DERIVATISATION OF GARCINIA ACID IN *GARCINIA ATROVIRIDIS*

By

HABIBAH ZAINAL ABIDIN

June 2005

Chairman : Professor Nordin Hj Lajis, PhD

Institute : Bioscience

A method for extraction and isolation of garcinia acid from *Garcinia atroviridis (G.atroviridis)* was developed. Using this method high yields of garcinia acid in the range of 90-95 g from 500 g of the dried rinds were accomplished. This method is low cost and simple. The garcinia acid isolated from this procedure was converted to its calcium salt of HCA lactone to ensure that the compound was stable and less hygroscopic. From the analysis using high performance liquid chromatography (HPLC), three peaks of organic acids were observed and identified as α-hydroxycitric acid lactone (HCA lactone), α-hydroxycitric acid (HCA) and citric acid based on spiking with standard acids and retention time. The amount of the HCA was 72 % w/w, the HCA lactone was 17 % w/w and the citric acid was 0.4 % w/w in the calcium salt of HCA lactone. The calcium salt of HCA lactone was derivatised into ester. The esterification products are dimethyl tetrahydro-3-hydroxy-5-oxo-2,3-furandicarboxylate and diethyl tetrahydro-3-hydroxy-5-oxo-2,3-furandicarboxylate. Both esters were tested for biological activity towards nitric oxide (NO). Unfortunately, both compounds were

found inactive towards NO inhibition RAW 264.7 cell treated with lipopolysaccharide (LPS) and interferon-γ (IFN-γ).

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENILAIAN, PENGELUARAN, DAN TERBITAN ASID GARSINIA DIDALAM *GARCINIA ATROVIRIDIS*

Oleh

HABIBAH ZAINAL ABIDIN

June 2005

Pengerusi : Profesor Nordin Hj Lajis, PhD

Institute : Biosains

Kaedah untuk pengekstrak dan pengasingan asid garsinia dari *Garcinia atroviridis (G.atroviridis)* telah dimajukan. Kaedah ini dapat menghasilkan asid garsinia yang tinggi didalam linkungan 90-95 g daripada 500 g asam keping yang kering. Kaedah ini didapati melibatkan kos yang rendah dan mudah. Asid garsinia yang telah diasingkan ini telah ditukarkan dalam bentuk garam kalsium HCA lakton untuk memastikan bahan tersebut didalam keadaan stabil dan kurang higroskopik. Tiga puncak asid organik telah didapati daripada analisis dengan menggunakan kromatografi cecair tekanan tinggi (KCTT) yang dikenalpasti sebagai α-asid hidroksisitrik lakton, α-asid hidroksisitrik dan asid sitrik berdasarkan sampel pakuan dengan asid piawai dan masa retensi. Kandungan asid hidroksisitrik adalah 72 % w/w, asid hidroksisitrik lakton adalah 17 % w/w dan 0.4 % w/w adalah asid sitrik didalam garam kalsium. Garam kalsium telah diesterifikasikan kepada ester. Produk esterifikasi adalah dimetil tetrahidro-3-hidroksi-5-okso-2,3-furandikarboksilat dan dietil tetrahidro-3-hidroksi-5-okso-2,3-furandikarboksilat. Kedua ester tersebut telah diuji terhadap aktiviti biologi nitrik oksida (NO). Kedua ester

didapati tidak aktif terhadap aktiviti biologi penahanan NO terhadap sel RAW 264.7 yang dirawat dengan lipopolysaccharide (LPS) dan interferon-γ (IFN-γ).

ACKNOWLEDGEMENTS

All praises to ALLAH the Almighty for giving me the strength to complete this study.

I would like to express my appreciation and thankfulness to my supervisor, Prof. Dr Nordin Hj Lajis, Assoc. Prof. Dr Khozirah Shaari and Assoc. Prof. Dr Daud Israf Ali for their guidance, encouragement, and support.

I would like to thank also to :

Ministry of Science, Technology and Environment for providing me a postgraduate scholarship throughout of this course.

Staffs and postgraduates students of the Institute of Bioscience, Universiti Putra Malaysia, especially Suryati and Maulidiani for their company and assistance throughout this study.

Staff of the Environments and Bioprocess Technology Centre at SIRIM Berhad especially to En Fadil Bin Mohamad for his assistance in running the ICP-OES.

Finally, I would like to thank to my husband, Abd Rahman Bin Bain, for his supports and understanding during the most difficult times. Last but not least to my lovely son, Ahmad Irwan Bin Abd Rahman.

I certify that an Examination Committee met on 22th June 2005 to conduct the final examination of Habibah Zainal Abidin on her Master of Science thesis entitled "The Evaluation, Production and Derivatisation of the Garcinia Acid in *Garcinia atroviridis*" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

MOHD ASPOLLAH HJ SUKARI, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Chairman)

ANUAR KASSIM, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Internal Examiner)

MOHD BASYARUDDIN AB RAHMAN, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Internal Examiner)

DATO' LAILY DIN, PhD
Professor
Centre for Graduate Studies
Universiti Kebangsaan Malaysia
(External Examiner)

GULAM RUSUL RAHMAT ALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 22 AUG 2005

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

NORDIN HJ LAJIS, PhD
Professor
Institute of Bioscience
Universiti Putra Malaysia
(Member)

KHOZIRAH SHAARI, PhD
Associate Professor
Institute of Bioscience
Universiti Putra Malaysia
(Member)

DAUD HJ ISRAF, PhD
Associate Professor
Institute of Bioscience
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD
Professor / Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 08 SEP 2005

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other at UPM or other institutions.

Habibah

HABIBAH ZAINAL ABIDIN

Date: 21 AUG 2005

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

min.	:	minute
HPLC	:	High Performance Liquid Chromatography
IR	:	Infra-red
GC-MS	:	Gas Chromatography Mass Spectrometer
LC-MS	:	Liquid Chromatography Mass Spectrometer
ICP-OES	:	Inductively Coupled Plasma–Optical Emission Spectrometer
HCA	:	Hydroxycitric acid
HCA Lactone	:	Hydroxycitric acid lactone
LOD	:	Limit of determination
H_2SO_4	:	Sulphuric acid
H_3PO_4	:	Phosphoric acid
NaOH	:	Sodium hydroxide
NO	:	Nitric oxide
G.atroviridis	:	*Garcinia atroviridis*
KCTT	:	Kromatografi Cecair Tekanan Tinggi
mp	:	melting point
LPS	:	lipopolysaccharide
IFN-γ	:	interferon-γ

CHAPTER 1

INTRODUCTION

Garcinia atroviridis Griff. ex T. Andes, a member of Guttiferae Family, is an endemic species in Peninsular Malaysia. It grows as a medium sized tree and bears yellow-orange little pumpkin-shaped fruit of which its dried form is widely used as seasoning. Its young leaves and shoots are eaten as salad. A decoction from the leaves and roots is used in the treatment of earache. The dried rinds of the fruits are also used in cooking (Burkill, 1966).

(-)-Hydroxycitric acid (1,2-dihydroxypropane-1,2,3- tricarboxylic acid, HCA) was encountered as the major organic acid in the rinds of *G. cambogia* fruits (Lewis et al., 1964; Lewis and Neelakantan, 1965). It has a similar chemical structure as citric acid (primary acid in citrus fruits) but contain two hydroxyl groups at C_α and C_β. HCA has been known to be a potent competitive inhibitor of ATP – citrate lyase and has been used for the control of obesity (Jena et al., 2002). In view of its wide medicinal uses, qualitative and quantitative evaluation of the acid in the fruit is established. The methods of production, extraction and derivatization are also developed to explore the potential of its commercial production.

HCA exists in two forms, the free tribasic acid and the dibasic acid lactone. HCA is easily converted into lactone during concentration and evaporation. HCA in its lactone form is stable but is inactive in inhibiting the citrate lyase as compared to free HCA. The stable derivatives of HCA, are in the forms of the sodium, potassium and calcium salts of HCA (Majeed et al., 1998).

Calcium salt of HCA lactone is isolated in large quantity from the dried rinds of the fruits of *G. atroviridis* using water extraction method. The modified method from the previously reported procedure was established by Ibnusaud et al., (2000) and Gokaraju et al., (2000). Water extraction method is used to minimize the use of organic solvent as well as for its lower cost. The compound was then derivatised into its stable and less hygroscopic calcium salt. The yield of calcium salt HCA lactone was reported to be higher than the yield of the HCA lactone (Gokaraju et al., 2000; Ibnusaud et al., 2000).

Three types of organic acid are determined in the dried rinds of *G. atroviridis* by high–performance liquid chromatography (HPLC) method. The organic acids are HCA, HCA lactone and citric acid. The main acid in the extract of the dried rinds of the fruits is HCA.

Previous studies on the HPLC method for the determination of organic acid were conducted on *G. cambogia, G. indica*, *G. cowa* and *G. pedunculata* (Jayaprakasha and Sakariah, 1998, 2000, 2002; Jayaprakasha et al., 2003). HCA was found to be the main acid content in various types of *Garcinia*.

The calcium salt of hydroxycitric acid lactone is esterified into diethyl and dimethyl esters. The compound is derivatised into esters and their biological activity was evaluated towards nitric oxide (NO). Previous study by Mackeen et al., (2000) on the fruits has resulted in the isolation of the α–HCA ester and lactones which are potent antimicrobials activity on *Cladosporium herbarum*.

The objectives of this study are:

1. To evaluate the (-)-HCA content in the fruits of *G. atroviridis*
2. To develop the most efficient methods in extracting of *garcinia* acid from *G. atroviridis*
3. To generate (-)-HCA derivatives
4. To test their biological activity

CHAPTER 2

LITERATURE REVIEW

2.1 Genus *Garcinia*

Guttiferae (alternative name Clusiaceae) is a plant family mainly found in the tropics and consists of 40 genera and 1000 species. In Peninsular Malaysia 4 genera including *Garcinia* (49 species), *Calophyllum* (45 species), *Mesua* (23 species) and Mammea (4 species) have been found in all kinds of habitat. *Garcinia* is a largest genus of trees or shrubs of the family Guttiferae. The best known fruit is manggis (*Garcinia mangostana*), asam gelugor (*Garcinia atroviridis*), cherupu (*Garcinia prainiana*) and mundu (*Garcinia dulcis*), (Whitmore, 1973).

Garcinia atroviridis (*G. atroviridis*) is a medium sized tree with a tall narrowly cylindrical or conical crown of up to 25 meter high. The trunk is fluted at the base. The bark is dull grey, and rather cracked and fissured in small pieces. The latex is rather watery, colorless, and scant. The leaves are narrowly oblong 5 -12 x 1 ¼ -3 inches, tapered at the apex with fleshy leathery and dark glossy green in color. They have fine dark wavy lines on the underside, pointing down with upcurled sides. The stalk is ½ - 1 inch long. The fruits are 2 ½ - 4 inches wide, nearly round, large and fluted with 12-16 ribs and grooves. The color of the ripening fruit is orange-yellow with the large brownish disc-like stigma ½-3/4 inches wide. Both petals and sepals are persistent at the base. The fully ripe fruit is separating into pegs seeds. The male flowers are in terminal cluster while the female flowers are solitary and large of 1 ¼-1 ¾ inches wide (Corner, 1988).

G. atroviridis is also known as "asam gelugor" in Malaysia. The fruit is brilliant yellow-orange, and depressed-globose. The fluted fruit has an acid juicy fruit wall, which is mainly used as a seasoning or sour relish (asam), not only fresh but also as dried rinds which is prepared by slicing and sun drying. The fruit can be eaten by itself if stewed with plenty of sugar (Burkill, 1966). Usually it is used in curries as a substitute for tamarinds.

2.2 Sources of (-)-hydroxycitric acid (HCA)

(-)-Hydroxycitric acid is found in the fruit rinds of certain species of *Garcinia* which include *Garcinia cambogia, Garcinia indica and Garcinia atroviridis* (Lewis and Neelakantan, 1965). These species were found in India and Sri Lanka (Jena et al., 1998). Some of these species were found in Malaysia, such as *G. atroviridis* and *G. cowa* (Corner, 1988).

The fruits of *G. cambogia, G. atroviridis* and *G. indica* are too acidic to be eaten raw. The dried rinds of the fruits are used as a condiment for flavouring curries in place of tamarind or lemon in India. It is popularly known as "Malabar tamarind" and commercially for "Colombo curing" of fish (Jena et al., 2002) (Table 1). A decoction of the fruit rind of *G. cambogia* is given for rheumatism and bowel complaints. It is also employed in veterinary medicine as a rinse for the disease of the mouth in cattle. *G. indica* is used as a garnish to give an acid flavor to curries and also for preparing syrups during hot months. The fruit is also anthelmintic and

useful in piles, dysentery tumor, pains and heart complaints. The syrup from the fruit juice is given in billous affections (Jena et al., 2002).

In Malaysia, the sun dried rinds of under ripe fruits of *G. atroviridis* are sold as a sour relish for use in curries in place of tamarind and for the dressing of fish. The fruit is also used as a fixative with alum in the dyeing of silk. A decoction from the leaves and roots is used in the treatment of earache. A lotion made from this fruit and vinegar, is used to rub abdomen of a woman after confinement. The juice made from the leaves is given to a woman for treatment after childbirth (Burkill and Haniff, 1970).

2.3 Identification of (-)-hydroxycitric acid (HCA) by paper chromatography

Lewis and Neelakantan (1965) isolated the principal acid in the fruit rinds of *G. cambogia* and identified it as (-)-HCA on the basis of chemical and spectroscopic studies. Identification and separation of the hydroxycitric acid on Whatman No 1. paper were performed using n-butanol/acetic acid/water (4:1:5) and n-propanol/formic acid/water (4:1:5). The spots were identified by spraying with 5% metavanadate. Upon saponification of the acid mixture with excess alkali and passing it through a column of ion exchange resins (zeocarb 215), the eluate showed only one lower spot (R_f = 0.32) corresponding to the free (-)-HCA. On concentration, the eluate gave only one upper spot (R_f = 0.46) corresponding to the lactone. The fruit extracts showed two predominant acid spots on chromatograms on two different solvent systems.

Table 1: Comparison of the physical properties of hydroxycitric acid (HCA) and lactones isolated from *Garcinia* (Jena et al., 2002)

Garcinia		
Property	Free acid	Lactone
Mp (^{0}C)		178
[α] ^{20} D(deg)	-20	+100
Crystal shape		needles
Solubility		High in alcohol and water, fair in ether
Paper chromatogram (R_f)		
Butanol/formic acid/water	0.24	0.42
Propanol/acetic acid/water	0.26	0.36
Butanol/acetic acid/water	0.32	0.46
Metavanadate spray (5%)	yellow	Reddish orange

2.4 Determination of (-)-hydroxycitric acid (HCA)

(-)-Hydroxycitric acid content was determined by titration method in the previous study. The end point was obtained when the acid is titrated with sodium hydroxide and phenolphthalein as an indicator. However the value of the acid content was the total acid in the fruits or dried rinds (AOAC, 1970). In this method the concentration of (-)-HCA, HCA lactone, and other acids content could not be determined separately.

Loweinstein and Brunengraber (1981) have developed a method to determine the hydroxycitrate content of the fruit of *G. cambogia* by gas chromatography (GC). Gas chromatographic estimation involves the conversion of acid to volatile silyl derivatives. An OV-17 GC column (3mx3mm id) was used and run at 145^{0}C using nitrogen as the carrier gas (40 ml/min) with an injection port temperature of 300^{0}C. (-)-Hydroxycitric acid lactone was the major constituent of the extract, and the